REMARKS

In view of the amendments to the claims and the following remarks, the Examiner is requested to allow claims 34, 45-52, 61, 62, 69, 77, 79 and new claim 81, the only claims pending and under examination in this application.

FORMAL MATTERS:

Claim 69 has been amended for clarity. Specifically, the recitation of "wherein said endogenous GPCR comprises a mutation in its amino acid sequence so as to render it constitutively active" has been replaced with "obtaining a constitutively activated form of said endogenous GPCR, wherein said constitutively activated GPCR comprises a mutation in its amino acid sequence that increases its constitutive activity relative to said endogenous GPCR". Claim 69 has also been amended to change the word "determining" to "analyzing".

Claim 77 has been amended to recite "obtaining a constitutively activated form of said endogenous orphan GPCR, wherein said constitutively activated GPCR comprises a mutation in its amino acid sequence that increases its constitutive activity relative to the endogenous orphan GPCR" (similar to Claim 69), thereby incorporating the subject matter of Claim 80. Claim 77 has also been amended to replace the word "comparing" in (c) with "analyzing" as well as to be consistent with the "obtaining" step amendment. Support for these amendments can be found throughout the specification, e.g., on page 5, lines 10-14 and lines 27-30; page 31, line 22 to page 32 line 4; and page 35 lines 19-21.

Claim 80 has been canceled in view of the amendment to Claim 77.

Claims 45, 52 and 79 have been amended to be consistent with the amendments to Claims 69 and 77.

Claim 81 is added and specifies that the claimed method "is performed in a laboratory or research setting". Support for this claim can be found throughout the specification, see, e.g., page 55, lines 2-12 (and continuing through to page 58); and page 63, lines 14-22. In addition, the experiments in the Examples all are "performed in a laboratory or research setting."

As no new matter is added by these amendments, entry by the Examiner is respectfully requested.

GRANTED CLAIMS IN CORRESPONDING EUROPEAN PATENT:

Applicants provide herewith a copy of claims that have been granted in the European counterpart to the subject application (Exhibit A). The granting of these claims, which mirror those in the subject application, indicates that the European Patent Office considers them to have clear industrial applications (i.e., to have utility).

INTERVIEW REQUEST:

Prior to filing the present Amendment and Response, Applicants requested an interview to discuss the outstanding utility rejection. However, the Examiner indicated that he and his supervisor considered that further discussion of the utility rejection would not be productive because they considered that the arguments supporting and rejecting utility of the claimed invention had been fully exhausted. Applicants are aware that prosecution of the present application has been long and that the utility issue has been difficult to resolve. However, Applicants consider that the issue of the utility of the claimed invention is one that has been constantly evolving and possibly nearing resolution.

Therefore, Applicants respectfully request that the Examiner, after review of the present amendment and response, reconsider extending the courtesy of an interview to discuss any remaining issue that is preventing allowance of the presently claimed invention.

REJECTIONS UNDER §101, UTILITY

Claims 34, 40, 45-66, 69 and 70 stand rejected under 35 U.S.C. §101 as lacking patentable utility.

In maintaining this rejection, the Office Action asserts that the claimed invention fails to meet the utility requirement because "the claimed methods lack a specific and substantial utility because there is no specific and substantial utility for a non-endogenous modulatory compound identified by the claimed method" (page 4).

The Office Action further cites MPEP §2107, which states that: "Utilities that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use are not substantial utilities... the following are examples of situations that require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use and, therefore, do not define "substantial utilities": (A) Basic research such as studying the properties of the claimed product

itself or the mechanisms in which the material is involved; (B) A method of treating an unspecified disease or condition; **(C) A method of assaying for or identifying a material that itself has no specific and/or substantial utility**" (emphasis added).

It appears to Applicants that item (C) above is being applied to the subject invention. Applicants respectfully traverse.

First, Applicants submit that the claimed invention is not drawn to "assaying for or identifying a material" as stated in item (C) above. Applicants submit that methods of "assaying **for** or identifying **a** material" (*emphasis added*) are those assays which merely detect the presence or absence of an analyte in a sample (and in the MPEP Guidance, the analyte itself would have no utility). This MPEP Guidance **is not** directed towards Applicants claimed invention. Specifically, Applicants submit that the phrase "assaying for or identifying a material" does not contemplate screening assays that analyze the functional activity of a compound as currently claimed. In the subject application, a plurality of candidate compounds are screened to find compounds that can modulate a particular orphan GPCR. This assay does not merely assay for or identify a material in a sample.

Thus, Applicants submit that, at least in one embodiment of the claimed invention, a different section of MPEP 2107 is applicable. Specifically, MPEP 2107.01(C), under the heading "Research Tools", states the following:

> C. Research Tools
> Some confusion can result when one attempts to label certain types of inventions as not being capable of having a specific and substantial utility based on the setting in which the invention is to be used. One example is inventions to be used in a research or laboratory setting. Many research tools such as gas chromatographs, **screening assays**, and nucleotide sequencing techniques have a clear, specific and unquestionable utility (e.g., they are useful in analyzing compounds).
> *(emphasis added)*

As explicitly stated above, research tools that are used in a research or laboratory setting, ***including screening assays as claimed in the present invention***, have "a clear, specific and unquestionable utility." In the case of the subject invention, the screening assay method identifies compounds that have modulatory activity on an orphan GPCR of interest. In one embodiment, these compounds can be employed in a predictable manner as reagents that have a known effect on the orphan GPCR (i.e., as agonists of inverse agonists).

Just as with sequencing assays, or, as argued previously, PCR assays, the user of the claimed screening assay determines which specific entity is the subject of the analysis (i.e., which specific orphan GPCR is to be employed to identify modulatory compounds). The reasons why a user wants to screen for modulatory compounds for a particular orphan GPCR will vary, including its activity in a specific cellular process (e.g., a disease process, like viral entry) as well as having an expression pattern of particular interest (e.g., in a specific diseased tissue or cells at a specific developmental stage). However, regardless of why a user is interested in a particular orphan GPCR, Applicants submit that the MPEP citation above clearly and explicitly states that screening assays have "a clear, specific and unquestionable utility."

Therefore, in view of the fact that the claimed screening assay is explicitly called out in MPEP 2107.01(C) as having "a clear, specific and unquestionable utility", Applicants submit that the claimed invention meets the utility requirements of 35 U.S.C. §101.

On page 6, the Office Action states that "the claims are rejected because they are directed solely to a method of identifying compounds for which there is no specific and substantial utility once identified. This is because the compounds modulate the activity of uncharacterized orphan receptors and this activity has not been associated with any particular, immediate use."

First, Applicants submit that a person skilled in the art would not make the effort to screen an orphan GPCR if they did not have some use for the compounds identified by the screening method. As detailed above, Applicants contend that the claimed screening assay has a specific and substantial "real world" use because it allows a user to identify, from a library of candidates, specific compounds that have a defined modulatory activity for an orphan GPCR of interest, regardless of the reason for why it is of interest to a user. This utility is clearly described throughout the application as providing researchers in the field with a novel approach to by-pass the significant bottle-neck in the orphan GPCR field, i.e., waiting for an orphan GPCR to be "de-orphanized" prior to conducting further functional studies. In view of this, Applicants submit that those of ordinary skill in the art would consider the claimed invention to have a particular and immediate use.

Second, Applicants contend that prior to the time of filing the present application, orphan GPCRs having a specific function or activity had been identified, and that modulatory compounds for such an orphan GPCR do indeed have specific and substantial utility. Fr example, Applicants have previously

submitted references which disclose orphan receptors STRL33, gpr1 and gpr15 as co-factors for retroviral entry into cells (see response dated November 13, 2007, and exhibits filed therewith).

Applicants submit that it is a common misperception that orphan receptors, and by extension compounds that modulate orphan receptors, have no utility. Knowledge of a GPCR's natural ligand is simply not necessary for establishing a useful function for such a receptor. In fact, it is possible to know a receptor's function and develop and market pharmaceutical agents targeting it without any understanding of the natural ligand which activates it. For example, many opiates were identified and developed and the analgesic functionality of these compounds at the mu-opiate receptor was appreciated long before the first endogenous agonists of that receptor were discovered in 1975 (see Zadina et al., Ann NY Acad Sci. 1999; 897:136-44, provided herewith as Exhibit B for the Examiner's convenience).

Therefore, because orphan GPCRs have been characterized, even in the absence of a known endogenous ligand, Applicants submit that identifying modulatory compounds for such functionally-characterized orphan GPCRs represents a specific, substantial "real world" use of the claimed invention.

On page 8, the Office Action asserts that "[t]here is no specific and substantial utility for any of the non-endogenous compounds identified by the claimed methods. Further research would be required to identify a use for any of the modulators identified by the claimed methods."

While not conceding this point, Applicants again stress that the claimed invention is **not** drawn to compositions of compounds but rather to screening assays for identifying modulatory compounds for an orphan GPCR of interest to a user. The question with regard to the utility of the claimed invention is thus whether practicing the subject methods provides a specific and substantial "real world" use. Applicants contend that it does.

In making this rejection, the Office Action essentially is asserting that more research is needed to identify a specific and substantial use for the compounds identified in the claimed screening method. Applicants disagree. Again, the subject claims are drawn to screening assays for identifying a compound having a specific activity, i.e., having a modulatory activity for an orphan receptor of interest to the user. Such compounds have as much immediate utility as would the endogenous ligand for the receptor. Specifically, as with the endogenous ligand, the compounds identified in the claimed screening assay can be employed in a predictable manner as reagents that have a known effect on the orphan GPCR (e.g., as agonists or inverse agonists). While performing further experiments on these

compound may be done (e.g., to identify a therapeutic application for one or more of the identified compounds) this is not required for the claimed screening methods to have utility.

Finally, in the paragraph spanning pages 8 and 9, the Office Action states that Applicants arguments likening the utility of the claims of US Patent 5,462,856 with the claimed invention were not persuasive because, unlike the '856 patent, "the claimed method is not directed to GPCRs in general, but is instead limited to orphan GPCRs that have no known ligand and which have no known activity that can be modulated for a useful purpose." The Office Action also notes that, regardless of Applicants arguments, the '856 patent was issued prior to publication of the revised Utility Examination Guidelines of 1/5/01 in the Federal Register.

First, Applicants submit that, in contrast to the assertion in the Office Action, orphan GPCRs have indeed been identified that have a known activity, e.g., the mu-opiate receptor discussed above.

Second, Applicants note that the '856 patent was issued ***after*** publication of the revised Utility Examination Guidelines of 1/5/01 (the '856 patent issued on 10/31/05).

Based on the discussion above, Applicants respectfully submit that the claimed invention has a significant and presently available useful benefit to the public. Applicants thus respectfully request withdrawal of this rejection under 35 U.S.C. §101.

REJECTIONS UNDER §112, ¶1 (ENABLEMENT)

Claims 34, 40 and 45-66, 69 and 70 stand rejected as not meeting the "how to use" part of the enablement requirement of 35 U.S.C. § 112, first paragraph.

The basis for this rejection is the Examiner's contention that the claims are not supported by a patentable utility.

As such, it is believed that this rejection has been adequately addressed in the discussion in the preceding section of this response.

In view of the discussion in the preceding section of this response, Applicants respectfully request withdrawal of this rejection.

REJECTIONS UNDER §102(b)

Claims 34, 45, 48, 61, 77 and 79 stand rejected under 35 U.S.C. 102(b) as being anticipated by Eggerickx et al (Biochem J. 309(Pt. 3): 837-843).

It will be appreciated that the standard for anticipation under section 102 is one of strict identity. An anticipation rejection requires a showing that each limitation of a claim be found in a single reference, *Atlas Powder Co. v. E.I. DuPont de Nemours & Co.*, 224 U.S.P.Q. 409, 411 (Fed. Cir. 1984). Further, an anticipatory reference must be enabling, see *Akzo N.V. v. United States Int'l Trade Comm'n* 808 F.2d 1471, 1479, 1 U.S.P.Q.2d 1241, 1245 (Fed. Cir. 1986), *cert denied*, 482 U.S. 909 (1987), so as to place one of ordinary skill in possession of the claimed invention. To anticipate a claim, a prior art reference must disclose every feature of the claimed invention, either explicitly or inherently. *Glaxo v. Novopharm, Ltd.* 334 U.S. P.Q.2d 1565 (Fed. Cir. 1995).

Claims 34, 45, 48, 61 and 79 depend from independent Claim 77. Applicants note that Claim 80, which also depends from Claim 77, was not rejected as anticipated by the Examiner over Eggerickx et al.

Applicants have thus amended Claim 77 to include the subject matter of Claim 80. Specifically, Claims 77 recites "obtaining a constitutively activated form of said endogenous orphan GPCR, wherein said constitutively activated GPCR comprises a mutation in its amino acid sequence that increases its constitutive activity relative to the endogenous orphan GPCR".

Because Eggerickx et al. fails to teach this element of amended Claim 77, Applicants submit that this reference cannot anticipate it or its dependents. Applicants respectfully request withdrawal of this rejection.

CONCLUSION

Applicants submit that the pending claims are in condition for Allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, he is invited to telephone the undersigned at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order numberAREN-001CIP.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: 10-8-08

By: _____
David C. Scherer, Ph.D.
Registration No. 56,993

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Enclosures:

1. Exhibit A: Copy of claims granted in European counterpart (8 pages);
2. Exhibit B: Zadina et al., Ann NY Acad Sci. 1999; 897:136-44 (9 pages)

F:\DOCUMENT\AREN\001CIP (001.US2.CIP)\001.US2.CIP (AREN-001CIP) Resp to OA dated 04-09-08.doc

EXHIBIT A

Europäisches Patentamt
European Patent Office
Office européen des brevets

European Patent Office
80298 MUNICH
GERMANY
Tel: +49 89 2399 0
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Application No.	Ref.	Date
98 918 196.1 - 2404	P021826EP	24.04.2008
Applicant: Arena Pharmaceuticals, Inc.		

Communication under Rule 71(3) EPC

You are informed that the Examining Division intends to grant a European patent on the basis of the above application with the text and drawings as indicated below:

In the text for the Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

Description, Pages

1-62, 64, 66-78, 80-88	as published
65	filed with telefax on 23.11.2005
63, 79	filed with telefax on 06.02.2008

Claims, Numbers

1-19	filed with telefax on 06.02.2008

Drawings, Sheets

1/17-17/17	as published

Comments
Art 84 EPC - Description has been adapted to the new set of claims (pages 24, 50 and 51) and figure 1 has been replaced by figure 2 and vice-versa (pages 4 and 24).

In the text for the Contracting States:
AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE

With the following amendments to the above-mentioned documents by the examining division

Description, Pages 4,24,50,51

A copy of the relevant documents is enclosed

The title of the invention in the three official languages of the European Patent Office, the international patent classification, the designated Contracting States, the registered name of the applicant and the bibliographic data are shown on the attached EPO Form 2056.

You are requested within a non-extendable period of **four months** of notification of this communication

1. to file 1 set of translations of the claim(s) in the two other EPO official languages;

		EUR
2a. to pay the fee for grant including the fee for printing up to and including 35 pages;	Reference 007	790.00
2b. to pay the printing fee for the 36th and each subsequent page; number of pages: 73	Reference 008	876.00
3. to pay the additional claim fee(s) (R. 71(6) EPC); number of claims fees payable: 0	Reference 016	0.00
	Total amount	1666.00

The mention of the grant of the patent shall be published in the European Patent Bulletin as soon as possible after the requirements concerning the translation of the claims and the payment of the fees for grant and printing, claims fees, designation fees and renewal fees as laid down in Rule 71(3), (4), (6) and (8) and (9) EPC are fulfilled.

Any divisional applications relating to this European patent application must be filed directly at the European Patent Office in Munich, The Hague or Berlin in accordance with Article 76(1) and Rule 36 EPC **before** the date on which the European Patent Bulletin mentions the grant of the patent (see Art. 97(3) EPC and OJ EPO 2/2002, 112).

If you do not approve the text intended for grant but wish to request amendments or corrections, the procedure described in Rule 71(4) EPC is to be followed.

If this communication is based upon an auxiliary request, and you reply within the time limit set that you maintain the main or a higher ranking request which is not allowable, the application will be refused (Art. 97(2) EPC).

If the enclosed claims contain amendments proposed by the Examining Division, and you reply within the time limit set that you cannot accept these amendments, refusal of the application under Article 97(2) EPC will result if agreement cannot be reached on the text for grant.

In all cases except those of the previous two paragraphs, if the fees for grant and printing or claims fees are not paid, or the translations are not filed, in due time, the European patent application will be deemed to be withdrawn (R. 71(7) EPC).

For all payments you are requested to use EPO Form 1010 or EPO Form 1010E or to refer to the relevant reference number.

After publication, the European patent specification can be downloaded free of charge from the EPO publication server https://publications.european-patent-office.org (OJ EPO 2005, 126).

Upon request in writing each proprietor will receive the certificate for the European patent **together with one copy** of the patent specification provided that the request is filed within the time limit of Rule 71(3) EPC. If such request has been previously filed, it has to be confirmed within the time limit of Rule 71(3) EPC. The requested copy is free of charge. If the request is filed after expiry of the Rule 71(3) EPC time limit, the certificate will be delivered without a copy of the patent specification.

Note on payment of renewal fees

If a renewal fee falls due between notification of the present communication and the proposed date of publication of the mention of the grant of the European patent, publication will be effected only after the renewal fee and any additional fee have been paid (R. 71(9) EPC).

Under Article 86(2) EPC, the obligation to pay renewal fees to the European Patent Office terminates with the payment of the renewal fee due in respect of the year in which the mention of the grant of the European patent is published.

Filing of translations in the Contracting States

Pursuant to Article 65(1) EPC the following Contracting States require a translation of the specification of the European patent in their/one of their official language(s) (R. 71(10) EPC), **if** this specification is not published in their/one of their official language(s)

- within **three months** of the publication of the mention of the grant:

AT	AUSTRIA	FR	FRANCE
BE	BELGIUM	GB	UNITED KINGDOM
CH	SWITZERLAND / LIECHTENSTEIN	GR	GREECE
CY	CYPRUS	IT	ITALY
DE	GERMANY	NL	NETHERLANDS
DK	DENMARK	PT	PORTUGAL
ES	SPAIN	SE	SWEDEN
FI	FINLAND		

- within **six months** of publication of the mention of the grant:

IE	IRELAND

The date on which the mention of the grant of the European patent will be published in the European Patent Bulletin will be indicated in the decision to grant the European patent (EPO Form 2006A).

The translation must be filed with the national Patent Offices of the Contracting or Extension States in accordance with the provisions applying thereto in the State concerned. Further details (e.g. appointment of a national representative or indication of an address for service within the country) are given in the

EPO information brochure "National law relating to the EPC" and in the supplementary information updates published in the Official Journal of the EPO, or are available on the EPO website.

Failure to supply such translation to the Contracting or Extension States in time and in accordance with the aforementioned requirements may result in the patent being deemed to be void ab initio in the State concerned.

Important note to users of the automatic debiting procedure

The fees for grant and printing and also any additional claims fees due under Rule 71(6) EPC will be debited automatically on the date of filing of the translation of the (relevant) claims, or on the last day of the period of this communication. However, if the designation fees become due as set out in Rule 71(8) EPC and/or a renewal fee becomes due as set out in Rule 71(9) EPC, these should be paid separately by another permitted means of payment in order not to delay the publication of the mention of grant. The same applies in these circumstances to the payment of extension fees. For further details see the Arrangements for the automatic debiting procedure (AAD) and accompanying information from the EPO concerning the automatic debiting procedure (Annexes A.1 and A.2 to the Arrangements for deposit accounts (ADA) in Supplement to OJ EPO 10/2007).

Examining Division:

Chairman:	Hinchliffe, Philippe
2nd Examiner:	Weijland, Albert
1st Examiner:	Pinheiro Vieira, E

Europäisches Patentamt • European Patent Office • Office européen des brevets

López Puche, Ruth
For the Examining Division
Tel. No.: +49 89 2399 - 7935

Enclosure(s): Form 2056
108 Copies of the relevant documents

+++ ATTENTION +++

New amounts of procedural fees as from 01.04.2008 (see OJ EPO 1/2008)!

If additional claims fees (R. 71(6) EPC)* are to be paid and payment is received on or after 01.04.2008, claims fees are only payable from the sixteenth claim onwards. New

amount to be paid: EUR 200,- per additional claim.
* to be amended

European Patent Application No. 98918196.1
Main Request

Claims:

1. An in vitro method for directly identifying a candidate compound as a compound that stimulates ann ~~inverse agonist, a partial agonist, or an agonist to a~~ orphan G-protein-coupled receptor, or a compound which acts to diminish the active state of the orphan G-protein-coupled receptor, wherein the orphan G protein-coupled receptor comprises a mutation in its amino acid sequence so as to render it ~~receptor is~~ a non-endogenous constitutively activated orphan G protein-coupled receptor, said method comprising the steps of

   (a) contacting said candidate compound with said non-endogenous constitutively activated orphan G protein-coupled receptor, wherein said orphan G protein-coupled receptor is expressed on a mammalian cell; and

   (b) determining, by measurement of the ability of the compound to inhibit or stimulate receptor functionality, wherein said candidate compound is a compound that stimulates said orphan G protein-coupled receptorn ~~inverse agonist, a partial agonist, an agonist~~ or a compound which acts to diminish the active state of said orphan G protein-coupled receptor.

2. The method of claim 1 wherein said mutation includes single amino acid mutations.

3. The method of claim 1 wherein said mutation is produced by using a mutational cassette.

4. The method of claim 1 wherein the compound is determined to be an inverse agonist to said receptor.

~~3. The method of claim 1 wherein the orphan receptor is a G protein coupled cell surface orphan receptor.~~

~~4~~5. The method of claim ~~3~~1 wherein the third intracellular loop of said orphan G protein-coupled receptor comprises the following sequences: X1BBHyX2 wherein X1 is an amino acid; B is a basic amino acid; Hy is a hydrophobic amino acid, and X2 is an amino acid.

~~5~~6. The method of claim 45 wherein X1 is glycine.

~~6~~7. The method of clam 45 wherein X1 is alanine.

~~7~~8. The method of claim 45 wherein X1 is lysine.

~~8~~9. The method of claim 45 wherein Hy is alanine

~~9~~10. The method of claim 45 wherein X2 is lysine

~~10~~11. The method of claim 45 wherein X2 is arginine.

~~11~~12. The method of claim 45 wherein X2 is glutamic acid

~~12~~13. The method of claim ~~3~~1 wherein the second intracellular loop of said orphan G protein-coupled receptor comprises the following sequences: XRY wherein X can be any amino acid other than D.

~~13~~14. The method of claim 45 wherein said sequence X1BBHyX2 is an endogenous sequence.

~~14~~15. The method of claim 45 wherein said sequence X1BBHyX2 is a non-endogenous sequence.

~~16~~16. The method of claim ~~12~~13 wherein the sequence XRY is an endogenous sequence.

~~16~~17. The method of claim ~~12~~13 wherein the sequence XRY is a non-endogenous sequence.

~~17~~18. A method according to any one of the preceding claims wherein the ability of the compound to inhibit or stimulate receptor functionality is detected by measuring the change in cAMP levels when said candidate compound is contacted with said constitutively activated orphan G protein-coupled receptor.

~~18~~19. A method according to any one of the preceding claims wherein the ability of the compound to inhibit or stimulate receptor functionality is detected by measurement of [^{35}S]GTPγS binding.

~~19. A method according to claim 1 further comprising the step of formulating the compound into a pharmaceutical composition.~~

Endomorphins: Novel Endogenous μ-Opiate Receptor Agonists in Regions of High μ-Opiate Receptor Density

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ABSTRACT: **Endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EM-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM-2) are peptides recently isolated from brain that show the highest affinity and selectivity for the μ (morphine) opiate receptor of all the known endogenous opioids. The endomorphins have potent analgesic and gastrointestinal effects. At the cellular level, they activate G-proteins (^{35}S-GTP γ-S binding) and inhibit calcium currents. Support for their role as endogenous ligands for the μ-opiate receptor includes their localization by radioimmunoassay and immunocytochemistry in central nervous system regions of high μ receptor density. Intense EM-2 immunoreactivity is present in the terminal regions of primary afferent neurons in the dorsal horn of the spinal cord and in the medulla near high densities of μ receptors. Chemical (capsaicin) and surgical (rhizotomy) disruption of nociceptive primary afferent neurons depletes the immunoreactivity, implicating the primary afferents as the source of EM-2. Thus, EM-2 is well-positioned to serve as an endogenous modulator of pain in its earliest stages of perception. In contrast to EM-2, which is more prevalent in the spinal cord and lower brainstem, EM-1 is more widely and densely distributed throughout the brain than EM-2. The distribution is consistent with a role for the peptides in the modulation of diverse functions, including autonomic, neuroendocrine, and reward functions as well as modulation of responses to pain and stress.**

DISCOVERY OF ENDOGENOUS OPIOIDS

The first endogenous agonists for opiate receptors (enkephalins[1]) were discovered in 1975. Examination of differences between morphine and enkephalins in their activities in bioassays led to the discovery of the δ-opiate receptor.[2] The enkephalins

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bound with greater affinity to the δ receptor than to the other two currently known opiate receptors, the μ and κ receptors. The enkephalins are therefore considered to be the endogenous agonists for the δ receptor. β-Endorphin, the second endogenous opiate-like agonist discovered,[3] was found to bind with about equal affinity to the μ and δ receptors.[4] Dynorphins, the last of the three currently well-known and characterized families of peptides, were discovered in 1979[5] and preferentially bind to κ receptors.[6]

A common motif in all of these peptides is the signature N-terminus tetrapeptide Tyr-Gly-Gly-Phe. Based to some extent on this sequence homology, each of the three peptide families can "spill over" to at least one of the receptors other than its "preferred" site. Thus, enkephalins bind to μ receptors at a 10–20-fold lower affinity than to their preferred δ receptor. Dynorphin binds with a sixfold lower affinity to the μ site than to the κ site, and β-endorphin is indiscriminate in its binding to μ or δ receptors.[4]

A guiding concept leading to the discovery of endogenous opioid peptides was that it seemed unlikely that a receptor would be present in the nervous system only to respond to a ligand (morphine) derived from a plant (poppy), to which an organism may or may not ever be exposed. A more likely possibility was that the nervous system would produce a natural agonist for that receptor. The discovery of opiate receptors in the brain[7] therefore stimulated the race to find the endogenous ligand, and within a short time, the peptides described above were discovered.

Based on a similar concept, a mystery remained after the discovery of the three families of opioid peptides. The μ-opiate receptor is essential to the analgesic and euphoric actions of morphine, as shown by the elimination of these effects in μ-opiate receptor knockout mice.[8,9] It seemed unlikely that the only natural ligands for the μ receptor were those that either preferentially bind to other types of opiate receptors (enkephalin to δ and dynorphin to κ receptors) or indiscriminately bind to μ or δ receptors (β-endorphin). The plant-derived alkaloid, morphine, by contrast, binds to the μ receptor with nearly two orders of magnitude greater affinity compared with its next-preferred site, the δ receptor.[4,10]

PEPTIDES SELECTIVE FOR μ RECEPTORS

Naturally occurring peptides have been found that preferentially bind to the μ opiate receptor. In contrast to the peptides discussed above, most of these peptides have an N-terminus Tyr-Pro sequence and include β-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) from tryptic digests of β-casein,[11] hemorphin (Tyr-Pro-Trp-Thr)[12] from digests of hemoglobin, Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2)[13] and Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2),[14] both isolated from brain. Although an aliphatic amino acid in the third position can confer selective binding to the μ receptor, as with Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2),[15] an aromatic (Trp or Phe) amino acid in this position provides higher affinity binding.[10] For all of these peptides, however, the affinity for the μ receptor was well below that of the familiar endogenous opioids. The highest affinity (20-50 nM) exogenous peptide was the casomorphin-derived peptide morphiceptin, whereas the highest affinity endogenous peptide was Tyr-W-MIF-1 (70 nM).[10]

EXHIBIT B

DISCOVERY OF ENDOMORPHINS

Based on knowledge of this "Tyr-Pro-aromatic" motif for μ selectivity, we used the brain peptide Tyr-W-MIF-1 (Tyr-Pro-Trp-Gly-NH_2) as a parent compound to search for a higher-affinity natural ligand.[16] We systematically substituted each of the 20 natural amino acids for the Gly in position 4 and tested these analogues for binding to the μ receptor. Although most of the analogues showed affinities in the range of 0.2–6 times higher than that of Tyr-W-MIF-1, the analogue with Phe in position 4 (Phe^4) showed an affinity of more than an order of magnitude higher than the other analogues. The selectivity for binding to the μ receptor relative to the δ or κ receptor also was dramatically increased, with an affinity ratio of more than three orders of magnitude. Charged amino acids inhibited the binding, but the hydrophobicity of the amino acid in position 4 correlated with binding. The binding of the Phe^4 analogue, however, showed a significant deviation from this correlation, indicating that the dramatic increase in binding could not have been predicted from theoretical models based on charge or hydrophobicity.

The high-affinity binding of this newly characterized peptide (Tyr-Pro-Trp-Phe-NH_2) did not necessarily mean it would have agonist properties. Furthermore, just because it was composed of natural amino acids did not mean that the nervous system produced it. The former issue was resolved by the demonstration that the peptide was extremely potent in the guinea-pig ileum assay, the classical test of opiate agonist activity.[16] It was more active than the potent enkephalin analogue DAMGO, and this effect was reversed by the μ antagonist CTOP, reflecting its selectivity for the μ receptor. The analogue also had potent and specific agonist action *in vivo* as shown in the tail-flick test. The antinociceptive potency after intracerebroventricular (i.c.v.) injection rivaled that of morphine and was reversed by the specific μ antagonist β-funaltrexamine. The peptide was even more potent after intrathecal (i.t.) injection than after i.c.v. injection.

The question of whether the nervous system could produce such a peptide was addressed by the generation of a specific antibody against it and use of that antibody to screen fractions of bovine brain extract purified by HPLC. After several steps of separation, a purified immunoreactive fraction was subjected to Edman degradation sequencing to reveal the presence of two peptide sequences: Tyr-Pro-Trp-Phe-NH_2 and Tyr-Pro-Phe-Phe-NH_2. With only slightly lower affinity than the first sequence, the second also was found to bind with subnanomolar affinity and >1000-fold selectivity for the μ receptor and to have potent agonist activity in the ileum assay and tail-flick test. Thus, the brain extract contained two previously unknown opioid peptides. Subsequently, similar isolation procedures with extracts of human brain[17] revealed that both peptides also were present in human brain and in greater quantities than in the bovine brain where they were originally discovered.[16]

One of the original names proposed for endogenous morphine-like compounds was the contraction "endorphin." This term was not chosen, however, as the name for the first opiate-like peptide discovered, enkephalin. Subsequently, β-lipoprotein was found to contain a sequence with potent opiate-like activity that was named β-endorphin.[3] Although it was proposed in 1983 by a committee of the prestigious International Narcotics Research Conference (INRC) to restrict the term endorphin to refer to the specific peptide β-endorphin, the term endorphins has commonly been

used within the scientific community as well as in the general public as a generic term for all of the endogenous opioids. This original contraction, proposed by Eric Simon, did not include an "m", in part to clearly distinguish the endogenous compounds from morphine. This may in retrospect have been appropriate: None of the three previously known peptide families bound preferentially to the μ receptor, although each of them could activate the site as described above. Because the two newly discovered peptides (Tyr-Pro-Trp-Phe-NH_2 and Tyr-Pro-Phe-Phe-NH_2) were endogenous peptides that had the highest affinity and selectivity for the μ receptor, they were named endomorphin-1 and -2 (EM1 and EM2).

CELLULAR ACTIONS OF ENDOMORPHINS

In addition to the *in vitro* effects in the ileum and the i.c.v. and i.t. effects on analgesia described in the original paper, post-receptor cellular actions of the peptides also were consistent with their μ agonist profile. A major effect of opioids is to inhibit cellular excitability. One mechanism by which this is achieved is by inhibition of calcium currents. When applied to neuroblastoma cells transfected with the human μ-opiate receptor, the endomorphins showed a dose-dependent, naloxone-reversible inhibition of voltage-dependent calcium channels.[18]

The μ receptor is a G-protein coupled receptor, so the signal transduction cascade of μ receptor agonists begins with activation of G-proteins. When applied to membranes of μ-receptor-containing SH-SY5Y human neuroblastoma cells, the endomorphins stimulate the binding of ^{32}S-GTP-γ-S to the membranes.[19] This relatively recent, but now well-established, test for activation of G-proteins also revealed that the endomorphins have lower efficacy than DAMGO, the standard high-efficacy μ agonist that is the reference compound in this assay. This difference in efficacy may have implications for the susceptibility of the agonists to desensitization. It may be, for example, that the lower efficacy of the endomorphins could make them more resistant to loss of responsiveness with repeated or prolonged exposure. The effect of endomorphins compared with DAMGO in the GTP-γ-S binding assay, however, is in contrast to that observed in the guinea-pig ileum, where the endomorphins were significantly more potent than DAMGO.[16] The basis for these differences is unclear at this time.

MAPPING THE DISTRIBUTION OF THE ENDOMORPHINS

Endomorphin-2 in the Spinal Cord and Brainstem

Although exogenous application of peptides in the various tests described thus far is very useful for the determination of their binding and agonist characteristics, an understanding of the natural endogenous role of the peptides depends upon a detailed characterization of their distribution in the nervous system. A crucial criterion for characterizing the peptides as endogenous agonists for the μ receptor is that the neuronal processes releasing them should be anatomically localized near neurons expressing the μ receptor. The initial report of the discovery of the endomorphins demonstrated by RIA that the peptides were present in areas important for μ ac-

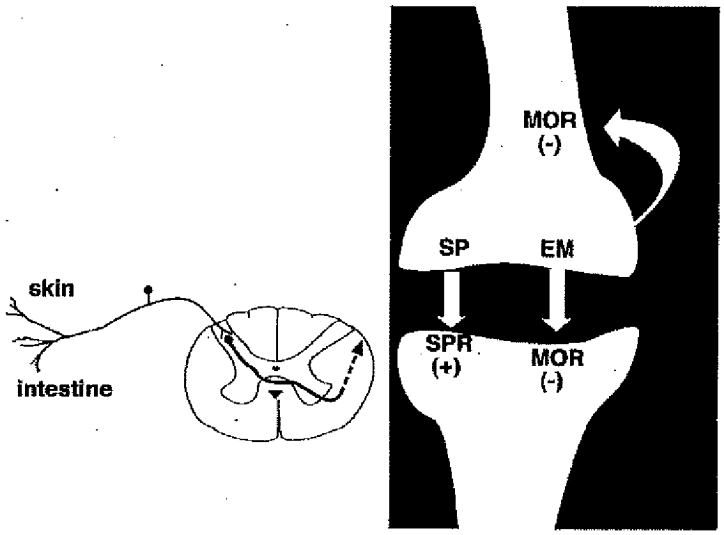

FIGURE 1. Hypothetical role of endomorphin (EM) in the modulation of nociceptive input at the synapse of primary afferents in the dorsal horn. Activation of presynaptic μ opiate receptors (MOR) could inhibit (–) the release of neurotransmitters such as substance P (SP), reducing the activation of the SP receptor (SPR) that normally has excitatory (+) effects on postsynaptic cells. EM could also act at postsynaptic MOR to decrease excitability in these cells.

tions.[16] The first detailed immunocytochemical demonstration that an endomorphin was present in close proximity to μ receptors showed relatively intense staining of EM2-like immunoreactivity (EM2-LI) in the superficial layers of the dorsal horn in the spinal cord and medulla and in fibers within the dorsal root ganglia.[20]

The dorsal horn has long been known to contain one of the highest densities of μ-opiate receptors in the nervous system and is an important site for the analgesic effects of morphine. Severing the primary afferents (rhizotomy) dramatically reduces, but does not eliminate, μ receptors in the dorsal horn.[21] This is consistent with the idea that μ receptors are present both on the dendrites of the cell bodies within the dorsal horn of the spinal cord, as well as on the terminals of the primary afferents that originate in the dorsal root ganglia. A long-standing question concerning these presynaptic receptors, however, is what natural agonist activates them. The dynorphin- and enkephalin-expressing neurons present in the dorsal horn make few synaptic connections with primary afferent axons containing μ receptors, and they most likely modulate nociceptive input by postsynaptic rather than presynaptic mechanisms (for review, see ref. 22). It also is unlikely that these opioids serve an autoregulatory function: enkephalin is not present in a significant population of primary afferents and is consistently unaffected by rhizotomy. The effects of rhizotomy on dynorphin are inconsistent, and dynorphin immunoreactivity that is colocalized with substance P (SP) is unaffected by rhizotomy (for reviews see refs. 22 and 23).

By contrast, a substantial body of evidence supports the idea that EM2 is present in primary afferents where it can serve both an autoregulatory function (by activating the μ receptors on primary afferents), as well as function to regulate the excitability of postsynaptic cells (by activating the μ receptors on cells within the dorsal horn). EM2-LI is colocalized with SP-LI in a subset of SP fibers,[20,22] and with CGRP-LI.[24] These are the two major excitatory peptide transmitters responsible for transmission of nociceptive signals from the primary afferents to the projection neurons and interneurons in the dorsal horn. EM2 is also colocalized with the μ receptor in some fibers in the dorsal horn.[22] Unilateral dorsal rhizotomy dramatically reduces EM2 immunoreactivity in the dorsal horn only on the rhizotomized side.[22,24] Capsaicin selectively activates and, in high doses, can ablate nociceptive primary afferent C- and A-δ fibers.[25] This selective neurotoxin virtually abolished EM2 staining in the dorsal horn of the spinal cord and medulla.[22] Thus, disruption of primary sensory afferents by mechanical (rhizotomy) or chemical (capsaicin) methods essentially abolished EM2-like immunoreactivity. This body of evidence indicates that EM2 could serve as the long-sought agonist for the presynaptic μ-opiate receptors on primary afferents. As illustrated in FIGURE 1, the release of EM2 could activate the presynaptic μ receptor to limit the release of excitatory transmitters such as SP. In addition, activation of postsynaptic μ receptors on interneurons and projection neurons within the dorsal horn could decrease the excitability of these neurons. Thus, EM2 may play a major role in the endogenous regulation of the transmission of nociceptive information.

Endomorphin-1 and -2 in Brain

The studies described above established EM2 as a μ agonist localized in circuits involved in the earliest stages of processing nociceptive information. Subsequent mapping studies detailed the immunoreactivity for EM2[26] and for both EM1 and EM2[27] throughout the nervous system. In general, both EM1- and EM2-LI are present in most areas where either was observed.[27] There are striking differences, however, in a few specific areas and in the general pattern of distribution: EM2-LI predominates in the spinal cord and in parts of the medulla. By contrast, EM1-LI is more prevalent in the brain and upper brainstem. There are regional differences between the peptides in the nucleus tractus solitarius (NTS), parabrachial nucleus, and the amygdala. In the NTS, there are large numbers of EM2-LI immunoreactive varicose fibers in the ventrolateral nucleus, whereas EM1-LI is present in cell bodies (visible without colchicine treatment) and in punctate terminal field elements in the dorsomedial nucleus. In the parabrachial nucleus, EM1-LI processes are primarily located in areas lateral to the superior cerebellar peduncle, whereas EM2-LI fibers are predominantly found ventral to it. In the amygdala, EM1-LI processes are present in all nuclei, whereas EM2-LI neuronal elements are relatively confined to the centrolateral nucleus. Thus, although the distributions of the two peptides are very similar, there are some striking differences, possibly indicating either separate precursors or differential processing of a single precursor.

The areas described above that are enriched in endomorphin-LI neuronal elements are also known to contain high densities of μ-opiate receptors and to be in-

volved in the processing of nociceptive information. Other brainstem areas that share these features include the periaqueductal gray, locus coeruleus, nucleus ambiguus, and caudal nucleus of the spinal trigeminal tract. These areas are known to receive primary afferents (e.g., visceral afferents to the NTS) and projection neurons from the dorsal horn, and to serve as relay nuclei to other pain-processing regions. Projection neurons from lamina I of the spinal cord, for example, terminate in the parabrachial nuclei, which in turn convey nociceptive information to the central nucleus of the amygdala. This spino(trigemino)ponto-amygdaloid pathway could modulate emotional/affective, behavioral, and autonomic reactions to noxious stimuli.[28] Endomorphin-containing neuronal elements are present in most regions of this pathway.

In addition to areas known to regulate pain, such as the amygdala and midline thalamic nuclei, diencephalic and telencephalic structures enriched in EM-LI indicate a role in neuroendocrine, homeostatic, and limbic functions. After i.c.v. injection of colchicine in the rat, the hypothalamus is the only region where cell bodies are detected except for the NTS (where EM1 cell bodies are found without use of colchicine). Both EM1- and EM2-immunoreactive cells are found in the posterior hypothalamus. Cell bodies for endomorphins may be restricted to these two areas, as β-endorphin cell bodies are restricted to the arcuate nucleus and NTS. It also is possible, however, that the restricted diffusion of colchicine after i.c.v. injection limited the detection of cell bodies to nuclei near the ventricles, and future studies may reveal additional cells that synthesize endomorphins.

Several telencephalic and limbic structures contain both EM-LI fibers and μ-opiate receptors. These include septal nuclei, the diagonal band of Broca, bed nucleus of the stria terminalis, the amygdaloid complex, and many hypothalamic nuclei. In the striatum, the striasomes are rich in μ receptors, but sparse in EM-LI. This area is associated with locomotor effects of opiates and provides a striking example of a mismatch between the μ receptor and endomorphins. At the ventral boundary of this region, however, the nucleus accumbens, which is associated with reward circuitry, contains both μ receptors and many EM1-LI fibers. Some species differences in the distribution of EM-LI exist. For example, EM1-LI fibers in the globus pallidus and cell bodies in the superior olive were detected in mouse but not rat. These results suggest the possibility of some differential functions of EM in the different species.

In summary, the endomorphins are high-affinity endogenous opioids with high selectivity for the μ-opiate receptor. They are potent analgesics and have cellular effects consistent with their μ-agonist profile. Their distribution in many regions of the nervous system containing μ receptors reflects a role as the natural agonists for this receptor. Modulation of pain, autonomic function, and stress responses are functions most likely implicated by the histochemical data, but numerous other functions, including homeostatic, neuroendocrine, and reward processes, also could be modulated by endomorphins.

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